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16. A DNA sequence that encodes a Coleopteran-type toxin protein of Bacillus thuringiensis var. tenebrionis which is effective in controlling Coleopteran-type insects having the amino acid sequence from residues (48-644) of said protein wherein the amino acid residues of said protein are numbered as shown in FIG. 10.

17. A transformed plant cell expressing the toxin protein of Bacillus thuringiensis var. tenebrionis having the amino acid sequence from residues (48-644) of the full-length protein wherein the amino acid residues of said full-length protein are numbered as shown in FIG. 10.

18. A transformed plant selected from the group consisting of tomato and potato expressing the toxin protein of Bacillus thuringiensis var. tenebrionis having the amino acid sequence from residues (48-644) of the full-length protein wherein the amino acid residues of said full-length protein are numbered as shown in FIG. 10.

19. A substantially pure toxin protein of Bacillus thuringiensis var. tenebrionis having the amino acid sequence from residues (48-644) of the full-length protein wherein the amino acid residues of said full-length protein are numbered as shown in FIG. 10.

20. A toxin protein of Bacillus thuringiensis var. tenebrionis free of other proteins of Bacillus thuringiensis var. tenebrionis said toxin protein having the amino acid sequence from residues (48-644) of the full-length protein wherein the amino acid residues of said full-length protein are numbered as shown in FIG. 10.

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Therefore, the potential for genetically engineered plants which exhibit toxicity or tolerance toward Coleopteran insects was foreseen if such plants could be transformed to express a Coleopteran-type toxin at an insecticidally-effective level. Agronomically important crops which are affected by Coleopteran insects include alfalfa, cotton, maize, potato, rape (canola), rice, tobacco, tomato, sugar beet and sunflower.

Although certain chimeric genes have been expressed in transformed plant cells and plants, such expression is by no means straight forward. Specifically, the expression of Lepidopteran-type *B.t.* toxin proteins has been particularly problematic. It has now been found that the teachings of the art with respect to expression of Lepidopteran-type *B.t.* toxin protein in plants do not extend to Coleopteran-type *B.t.* toxin protein. These findings are directly contrary to the prior teachings which suggested that one would employ the same genetic manipulations to obtain useful expression of such toxins in transformed plants.

In accordance with one aspect of the present invention, there has been provided a method for producing genetically transformed plants which exhibit toxicity toward Coleopteran insects, comprising the steps of:

(a) inserting into the genome of a plant cell susceptible to attack by Coleopteran insects a chimeric gene comprising:

i) a promoter which functions in plant cells to cause production of RNA;

expression to result in the production of an effective amount of toxin protein to render the plant toxic to Coleopteran insects. Those skilled in the art recognize that the amount of toxin protein needed to induce the desired toxicity may vary with the particular Coleopteran insects to be protected against. Accordingly, while the CaMV35S, ssRUBISCO and MAS promoters are preferred, it should be understood that these promoters may not be optimal promoters for all embodiments of the present invention.

The mRNA produced by the chimeric gene also contains a 5' non-translated leader sequence. This sequence may be derived from the particular promoter selected such as the CaMV35S, ssRUBISCO or MAS promoters. The 5' non-translated region may also be obtained from other suitable eukaryotic genes or a synthetic gene sequence. Those skilled in the art recognize that the requisite functionality of the 5' non-translated leader sequence is the enhancement of the binding of the mRNA transcript to the ribosomes of the plant cell to enhance translation of the mRNA in production of the encoded protein.

The chimeric gene also contains a structural coding sequence which encodes the Coleopteran-type toxin protein of *Bacillus thuringiensis* or an insecticidally-active fragment thereof. Exemplary sources of such structural coding sequences are *B.t. tenebrionis* and *B. t. san diego*. Accordingly, in exemplary embodiments the present invention provides a structural coding sequence from *Bacillus thuringiensis* var. *tenebrionis* and insecticidally-active fragments thereof. Those skilled in the art will recognize that other structural coding sequence substantially homologous to the toxin coding sequence of *B.t.t.* can be utilized

This comparison was performed using the computer program BESTFIT of Devereux et al (1984) which employs the algorithm of Smith and Waterman (1981). BESTFIT obtains maximum alignment of two nucleotide or amino acid sequences. BESTFIT calculates two parameters, quality and ratio, which can be used as alignment metrics when comparing different alignments. Ratio varies between 0 and 1.0. A larger ratio indicates a better alignment (greater similarity) between two sequences.

The BESTFIT alignment shows that the two types of toxin genes are related at both the nucleotide sequence and amino acid sequence level. However, the alignment also shows that the two sequences are clearly distinct and possess many regions of mismatch at both the nucleotide and amino acid sequence levels. For example, the ratio for comparison of the two amino acid sequences is only 0.22. At the nucleotide sequence level, maximum alignment is obtained only by the introduction of many gaps in both sequences, and the ratio is only 0.072.

There are many sequenced examples of Leptidopteran-type toxin genes; similar comparison among these genes has shown that the gene from *B.t. kurstaki HD-1* described by Schnepf et al. (1985) and that from *B.t. kurstaki HD-73* described by Adang et al. (1985) represent the two most divergent Lepidopteran-type toxin genes. By comparison with the ratios calculated above for alignment of the Colepteran-type and the Lepidopteran-type gene, the ratio for amino acid sequence comparison of the two most divergent Lepidopteran-type proteins is 0.811, and the ratio for these two Lepidopteran-type genes at the nucleotide sequence level is 0.755. This indicates that although the

agitating them for 20 min. in a 30% Chlorox solution containing 2 drops of Tween 20 per 400 mls before rinsing them twice with sterile distilled water. The seeds are then soaked in 0.4% benolate for 10 min. The benolate is poured off prior to placing the seeds aseptically onto agar solidified half strength MS salts. Seeds are germinated for 3-10 days in the dark at 32°C. The cotyledons and hypocotyls are then removed aseptically and segmented. The segments are placed onto 1) agar solidified MS medium containing 3% glucose, 2 mg/l napthalene acetic acid (NAA), and 1 mg/l kinetin (Medium MSS) or 2) Gelrite solidified MS medium containing 3% glucose, B5 vitamins, 100 mg/l inositol, 0.75 mg/l $MgCl_2$, 0.1 mg/l dichlorophenoxy acetic acid (2,4-D) and 0.1 or 0.5 mg/l kinetin (Medium MST). Callus is maintained in a 16/8 photoperiod at 28°C on either of these media until embryogenesis is initiated. Subculture of the embryogenic callus is made onto the same medium as for initiation but containing 3% sucrose instead of glucose. Somatic embryos are germinated by moving them onto Gelrite solidified Stewart's medium without plant growth regulators but containing 0.75 g/l $MgCl_2$. Germinated embryos are moved to soil in a growth chamber where they continue to grow. Plants are then moved to the greenhouse in order to set seed and flower.

Transformation of cotton tissues and production of transformed callus and plants is accomplished as follows. Aseptic seedlings are prepared as for plant regeneration. Hypocotyl and cotyledon segments are inoculated with liquid overnight *Agrobacterium* cultures or with *Agrobacterium* grown on nutrient plates. The explants are co-cultured for 2-3 days on MSS or MST medium containing 1/10 the concentration

Preparation of Maize Protoplasts

Protoplasts are prepared from a Black Mexican Sweet (BMS) maize suspension line, BMSI (ATCC 54022) as described by Fromm et al. (1985 and 1986). BMSI suspension cells are grown in BMS medium which contains MS salts, 20 g/l sucrose, 2 mg/l (2,4-dichlorophenoxy) acetic acid, 200 mg/l inositol, 130 mg/l asparagine, 1.3 mg/l niacin, 0.25 mg/l thiamine, 0.25 mg/l pyridoxine, 0.25 mg/l calcium pantothenate, pH 5.8. Forty ml cultures in 125 ml erlenmeyer flasks are shaken at 150 rpm at 26°C. The culture is diluted with an equal volume of fresh medium every 3 days. Protoplasts are isolated from actively growing cells 1 to 2 days after adding fresh medium. For protoplast isolation cells are pelleted at 200 X g in a swinging bucket table top centrifuge. The supernatant is saved as conditioned medium for culturing the protoplasts. Six ml of packed cells are resuspended in 40 ml of 0.2 M mannitol/50 mM $CaCl_2$/10 mM sodium acetate which contains 1% cellulase, 0.5% hemicellulase and 0.02% pectinase. After incubation for 2 hours at 26°C, protoplasts are separated by filtration through a 60 µm nylon mesh screen, centrifuged at 200 X g, and washed once in the same solution without enzymes.

Transformation of Maize Protoplasts with *B.t.t.* Toxin Gene DNA Vectors Using an Electroporation Technique

Protoplasts are prepared for electroporation by washing in a solution containing 2 mM potassium phosphate pH 7.1, 4 mM calcium chloride, 140 mM sodium chloride and 0.2 M mannitol. After washing, the protoplasts are resuspended in the same solution at a concentration of 4 X 10^6 protoplasts per ml. One-half

ml of the protoplast containing solution is mixed with 0.5 ml of the same solution containing 50 micrograms of supercoiled plasmid vector DNA and placed in a 1 ml electroporation cuvette. Electroporation is carried out as described by Fromm et al. (1986). As described, an electrical pulse is delivered from a 122 or 245 microFarad capacitor charged to 200 V. After 10 min. at 4°C and 10 min. at room temperature protoplasts are diluted with 8 ml of medium containing MS salts 0.3 M mannitol, 2% sucrose, 2 mg/l 2,4-D, 20% conditioned BMS medium (see above) and 0.1% low melting agarose. After 2 weeks in the dark at 26°C, medium without mannitol and containing kanamycin is added to give a final kanamycin concentration of 100 mg/l liquid. After an additional 2 weeks, microcalli are removed from the liquid and placed on a membrane filter disk above agarose solidified medium containing 100 mg/l kanamycin. Kanamycin resistant calli composed of transformed maize cells appear after about 1-2 weeks.

Expression of *B.t.t* Toxin Genes in Maize Cells

As described by Fromm et al. (1986), transformed maize cells can be selected by growth in kanamycin containing medium following electroporation with DNA vectors containing chimeric kanamycin resistance genes composed of the CaMV35S promoter, the NPTII coding region and the NOS 3' end. pMON9791 and pMON9792 contain such chimeric NPTII genes and also contain chimeric *B.t.t.* toxin genes. As described above, maize protoplasts are transformed by electroporation with DNA vectors where the DNA vectors are pMON9791 or pMON9792. Following selection for kanamycin resistance, the transformed maize cells are assayed for expression of the *B.t.t.* toxin gene.